Factors Associated with Fecal Shedding of Verotoxin-Producing *Escherichia coli* O157 on Dairy Farms

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ABSTRACT

Fecal samples were collected from 4,361 dairy cows on 91 dairy operations between 26 February and 8 July 1996. Fecal samples were cultured for *Escherichia coli* O157, and positive isolates were probed for verotoxin-producing genes. A total of 52 (1.2%) fecal samples on 22 (24.2%) operations were positive for verotoxin-producing *E. coli* O157. Herds in which samples were collected on or after 1 May 1996 were significantly more likely to test positive than herds sampled before that date (odds ratio = 7.7). Herds maintained on farms on which alleyways were flushed with water to remove manure were 8.0 times more likely to have samples test positive for verotoxin-producing *E. coli* O157 than were herds maintained on farms cleaned by use of other methods of manure removal.

Increased public concern about food safety has led to a desire to reduce the prevalence of foodborne pathogens along the entire food chain from farm to table. Verotoxigenic *Escherichia coli* O157 is an important human pathogen, causing up to 16,000 foodborne illnesses and 900 deaths per year in the United States, with an estimated annual cost of $200,000 to $600,000 *(2)*. A 1997 *E. coli* O157 outbreak resulted in over 1 million pounds of ground beef being recalled. A single outbreak in 1993 in the western United States caused over 700 illnesses and four deaths.

Sources of human exposure include direct contact with infected animals or people or consumption of contaminated meat, fruits, vegetables, water, or unpasteurized milk. One source of *E. coli* O157 is cattle. A beef feedlot study found positive fecal samples in 63% of feedlots tested, with 1.8% of fecal samples testing positive *(13)*. Since most of the milk that is consumed in the United States is pasteurized, the primary source of human exposure from dairy cattle is the culled cow. Dairy cows contribute 25% of nonfed beef available for consumption in the United States *(22)*.

E. coli O157 is believed to be widespread on U.S. dairy farms, but at very low prevalence *(9, 27)*. As such, this organism is not amenable to traditional eradication methods such as testing and quarantine or slaughter. Therefore, control measures depend on many factors, such as proper cooking and handling by consumers, proper handling during transport and by retail outlets, following hazard analysis critical control point procedures in slaughter plants to reduce fecal contamination of the carcass and meat products, and managing farms in such a way as to reduce fecal shedding. However, very little is known about the ecology of this organism on farms. Therefore, it is essential to identify management practices that are related to an increased prevalence of *E. coli* O157 so that intervention strategies can be developed that could reduce fecal shedding. The objectives of this study were to estimate the prevalence of fecal shedding of verotoxigenic *E. coli* O157 in dairy cows and to identify risk factors for *E. coli* O157 shedding on farms.

MATERIALS AND METHODS

Data collection. A stratified random sample from a list frame maintained by the National Agricultural Statistics Service was used to identify dairy herds for participation in the National Animal Health Monitoring System Dairy '96 study. Nearly 1,000 producers in the 20 largest dairy-producing states (Table 1) participated in the study. From this sample, a convenience sample of 50 small herds (herds with between 30 and 99 milk cows) and 50 large herds (herds with 100 or more milk cows) were invited to participate in the fecal sampling portion of the study. The number of small and large herds chosen to represent each state was proportional to the number of small and large herds in that state. Questionnaires were used to collect farm-level data regarding key hypothesized factors related to *E. coli* O157 shedding within the areas of inventory, housing, biosecurity, manure handling, and feeding practices.

Fecal samples were collected via rectal retrieval. Small farms were visited one time to collect fecal samples. All cull cows (defined as cows expected to be culled within the next 7 days) were sampled, and the remaining samples were from milk cows (lactating cows, dry cows, and cows in the sick pen), for a total of 40 samples. Cows were selected to represent the distribution of cows within herd in terms of parity and classification (lactating, dry, or sick). Large farms were visited three times. Fecal samples were collected from up to 20 cull cows during each visit. On one of these visits, an additional 50 fecal samples were collected from milk cows, again selected to represent the distribution of cows within herd in terms of parity and classification.

Laboratory methods. A new glove was used to collect each sample to avoid cross-contamination during sampling. Samples

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TABLE 1. *Participating states*

California	New Mexico
Florida	New York
Idaho	Ohio
Illinois	Oregon
Indiana	Pennsylvania
Iowa	Tennessee
Kentucky	Texas
Michigan	Vermont
Minnesota	Washington
Missouri	Wisconsin

TABLE 2. *Prevalence of Shiga-like toxin-positive* Escherichia coli O157 *in farm dairy cows*

	Samples		Herds	
Animal type	n	Positive (%)	n	Positive (%)
Milk cow	3,692	0.9	91	18.7
Cull cow	669	2.8	58	17.2
Total	4,361	1.2	91	24.2

were placed in sterile screw-top vials and shipped on ice overnight to the National Veterinary Services Laboratories in Ames, Iowa, for culturing. Approximately 1 g of each fecal specimen was added to 10 ml of modified *E. coli* broth *(20)*. The suspension was vortexed and incubated overnight at 35°C. A 96-well microtiter plate, containing 180 µl per well of trypticase soy broth (Difco Laboratories, Detroit, Mich.) in rows B through H, was used to make dilutions of the fecal suspension. Supernatants of modified *E. coli* broth cultures were added to each well of row A. Tenfold dilutions were made by transferring 20 µl sequentially between rows, resulting in two rows each of 10^{-2} and 10^{-4} dilutions and one row each of 10^{-1}, 10^{-3}, and 10^{-5} dilutions. From 10^{-2} and 10^{-4} dilutions, 300 µl were spread onto plates containing sorbitol–MacConkey agar with tellurite (Sigma Chemical Co., St. Louis, Mo.) and cefixime (Lederle, Pearl River, N.Y.). Plates were incubated at 35°C overnight. Up to 10 sorbitol-negative colonies picked from the plates were streaked onto sorbitol–MacConkey and MacConkey agars and stabbed into paranitrophenyl β-D-glucuronic acid agar (PGUA), followed by overnight incubation at 35°C *(4)*. Sorbitol-negative, lactose- positive, PGUA-negative, or weakly positive colonies were tested for indole. Indole positive colonies were serotyped to determine somatic (O) and flagellar (H) antigens *(26)*.

Colonies were transferred to trypticase soy broth and incubated at 37°C overnight. Antigens were prepared by heating cultures to 100°C for 1 h. One drop of diluted O157 rabbit antiserum (1:5 in normal saline with 0.5% phenol) was mixed with 2 to 3 drops of the prepared antigen in one well of a disposable multiwell tray. In another well, 1 drop of diluted negative rabbit serum (1:5 in phenolized saline) was mixed with 2 to 3 drops of antigen. Reactions were read after shaking 8 min. The isolate was considered to be O157 positive if agglutination occurred in O157 antiserum but not in negative serum. If no agglutination occurred in either well, the isolate was reported to be O157 negative. If both wells were positive, the isolate was considered to have a rough O antigen.

O157-positive isolates were transferred to the National Animal Disease Center in Ames, Iowa, for gene probing. The CVD 419, *eae, slt*I, *slt*II, and STaP DNA probes have been described *(15, 16, 18, 19)*. Probe DNA was labeled with a-^{32}P deoxycytidine (ICN, Costa Mesa, Calif.) using the large fragment of DNA polymerase I and random oligonucleotides as primers (Pharmacia, Piscataway, N.J.). Colony blots were prepared, and probe hybridization, filter washing, and X-ray film exposure were performed as previously described *(17)*. The colony blots were reprobed after stripping with 0.5 M NaOH *(17)*.

Data analysis. Samples containing *E. coli* isolates that expressed the O157 antigen and that were also positive for at least one verotoxin (SLT-I or SLT-II) were considered positive (VT-O157). Samples containing only rough O isolates were considered negative because the O157 antigen could not be confirmed. Anal-

ysis of herd-level risk factors was based on a dichotomous outcome (i.e., a herd was positive or negative for VT-O157). A herd was considered positive if at least one cow in the herd tested positive. Potential herd-level risk factors were screened one at a time via logistic regression, with VT-O157 status of the herd as the outcome variable. Because management and feeding practices varied by geographic region and season, region (north versus south) and time of year of sample collection were included as covariates in the screening process. Time of year was dichotomized into spring (26 February to 30 April) and summer (1 May to 8 July). Large herds that spanned the season break were categorized based on the time of year the majority of samples were collected. Herd size category was also included as a covariate to account for the sampling protocol that called for repeat visits to large herds to sample cull cows. Variables with a *P* value of less than 0.2 by this screening test were included in a backward elimination logistic regression multivariable modeling, with region, season, and herd size group as covariates. A *P* value of less than 0.05 was required to remain in the model. Odds ratios were determined from the coefficients in the logistic regression. The goodness of fit of the model was determined using the Hosmer–Lemeshow statistic. Population-attributable risk was calculated according to methods described by Bruzzi et al. *(1)*.

RESULTS

Attributes of the study herds. The owners of a total of 46 large herds and 45 small herds agreed to allow their herds to undergo fecal sample testing for VT-O157. Nearly all (95.6%) of the participant herds had Holstein cows. Over half of the study herds participated in Dairy Herd Improvement Association programs and were fed total mixed rations (60.4% and 52.7%, respectively). The mean rolling herd average milk production was 8,370 kg.

Herd-level results. Overall, 52 of 4,361 (1.2%) cows tested on farm were positive for VT-O157. The prevalence for milk cows and cull cows was 0.9% and 2.8%, respectively (Table 2). Of the 91 herds included in the study, 22 (24.2%) had at least one cow test positive for VT-O157 (Table 2). Of 45 small herds, only four herds were positive (8.9%). Nineteen small herds submitted cull cow samples, but none were positive. Of the 46 large herds, 18 (39.1%) were positive. Thirty-nine large herds submitted cull cow samples; five herds had both milk cows and cull cows testing positive, six herds had positive milk cows only, and five herds had positive cull cows only. In addition, two of the seven large herds that submitted only milk cow samples were positive.

Results of the screening analysis are shown in Table 3 (by environmental and management factors) and Table 4 (by feed ingredients). Shedding prevalence was higher in herds sampled during the summer months (52.9%) com-

TABLE 3. *Herds positive for* Escherichia coli *O157 (%) by various herd-level environmental and management factors*

Factor	Level	n	Positive (%)	Univariate P value	Covariate P value[a]
Season[b]	Spring	57	7.0	0.001	0.007
	Summer	34	52.9		
Geographic region	North	70	12.9	0.008	0.09
	South	21	61.9		
Herd size (no. of milk cows)	<100	45	8.9	0.001	0.40
	≥100	46	39.1		
Alley flush	Yes	19	68.4	0.001	0.01
	No	72	12.5		
Gutter cleaner	Yes	32	6.3	0.003	0.19
	No	59	33.9		
Cattle brought on the operation	Yes	52	30.8	0.09	0.10
	No	39	15.4		
Manure spread on land via irrigation	Yes	20	50.0	0.001	0.07
	No	68	13.2		
Manure spread on land via broadcast	Yes	78	19.2	0.13	0.29
	No	10	40.0		
Manure spread on land as slurry (on surface)	Yes	27	29.6	0.22	0.35
	No	61	18.0		
Cows drink from water tanks	Yes	75	29.3	0.01	0.22[c]
	No	16	0		
Cows drink from individual waters	Yes	32	12.5	0.06	0.69
	No	59	30.5		
Water chlorinated	Yes	13	15.4	0.42	0.41
	No	78	25.6		
Feed total mixed ration	Yes	48	33.3	0.03	0.49
	No	43	14.0		
Cows eat roughage or graze where manure was spread	Yes	46	28.3	0.36	0.78
	No	45	20.0		
Other species have access to feed	Yes	50	22.0	0.59	0.96
	No	41	26.8		
Lactating cows housed in tie stall, stanchion, individual	Yes	30	10.0	0.03	0.74
	No	61	31.1		

[a] Each variable analyzed individually via logistic regression with season of collection, geographic region, and herd size group as covariates.

[b] Spring includes 26 February to 30 April; summer includes 1 May to 8 July.

[c] Chi-square test limited to northern herds tested during the spring.

pared to herds sampled during the spring (7.0%). Herds in southern states had a higher prevalence (61.9%) than herds in the north (12.9%). Although herd size prevalences differed significantly on bivariate analysis, these differences were not significant after adjusting for region and season. However, this variable was included as a covariate to ac-

count for the different sampling schemes among herd size groups.

All herds in the southern region, and all but two herds tested during the summer, used water tanks for cows' drinking water. Therefore, this variable could not be modeled with region and season. A chi-square test was performed

TABLE 4. *Herds positive for* Escherichia coli *O157 (%) by various feed ingredients*

Feed ingredient	Level	n	Positive (%)	Univariate P value	Covariate P value[a]
Soybean	Yes	74	24.3	0.95	0.07
	No	17	23.5		
Meat–bone–blood meal	Yes	31	12.9	0.07	0.11
	No	60	30.0		
Cottonseed (meal, hulls, or whole)	Yes	55	36.4	0.001	0.12
	No	36	5.6		
Probiotics	Yes	19	42.1	0.04	0.18
	No	72	19.4		
Bakery products	Yes	12	25.0	0.94	0.28
	No	79	24.1		
Clover	Yes	28	21.4	0.68	0.37
	No	63	25.4		
Tallow	Yes	24	25.0	0.94	0.39
	No	66	24.2		
Brewers products	Yes	43	20.9	0.49	0.41
	No	48	27.1		
Corn silage	Yes	64	20.3	0.19	0.68
	No	27	33.3		
Alfalfa	Yes	62	21.0	0.30	0.94
	No	29	31.0		

[a] Each variable analyzed individually via logistic regression with season of collection, geographic region, and herd size group as covariates.
[b] Spring includes 26 February to 30 April; summer includes 1 May to 8 July.
[c] Chi-square test limited to northern herds tested during the spring.

on a subset of operations (northern herds tested during the spring); the test did not reveal a significant association between VT-O157 shedding and use of water tanks.

In addition to region, season, and herd size category, variables selected for logistic regression modeling included introduction of new cattle onto the operation, manure removal by gutter cleaner, manure removal by alley flush, manure spread via irrigation, manure spread via slurry, and feeding of cottonseed, soybean, meat–bone–blood meal, or probiotics. Results following a backward elimination process to eliminate variables from the model are shown in Table 5. The model fit the data well (Hosmer–Lemeshow goodness-of-fit statistic = 1.1, P = 0.89).

From the multivariable logistic regression analysis, herds in which manure was removed from the cow housing area by flushing the alleyways with water were eight times more likely to have a positive sample than herds using other manure removal systems (Table 5). The population attributable risk for alley flushing of manure was 0.517.

No association was found between herd status for VT-O157 and type of housing, access of other species of animals to feed, rodent control methods, manure application

to fields, probiotic use, water chlorination, or any of the feed ingredients evaluated.

DISCUSSION

Previous studies have demonstrated that herd shedding status can change from one sampling time to another (7, 11, 27). One study found that herds that tested positive once are more likely to test positive on a second sampling than are previously negative herds (7), suggesting that some herds have a higher prevalence than others and thus have a higher probability of detection. Yet most if not all dairy herds will eventually test positive for VT-O157 if enough samples are collected over time (12). Therefore, it was not the intent of this study to label herds "free" versus "infected" with VT-O157. The sampling protocol was designed to detect shedding at ≥5% prevalence within herds on a given day with at least 95% confidence (3). Risk factors were evaluated for detection at this level of infection. Assuming that higher-prevalence herds were more likely to be detected than lower-prevalence herds at any point in time, our objective was to evaluate differences in management and feeding practices between higher-prevalence

TABLE 5. *Results of backward elimination logistic regression for herd-level risk factors for VT-O157 fecal shedding*

Factor	Odds ratio	P values
Season		0.006
Summer	7.7	
Spring	1	
Geographic region		0.30
South	2.3	
North	1	
Herd size		0.81
<100 cows	1.2	
≥100 cows	1	
Flush alleyways with water to remove manure		0.01
Yes	8.0	
No	1	

herds (i.e., detectable) and lower-prevalence herds (i.e., not detectable).

Large herds were revisited to collect additional samples from cull cows in order to evaluate cull status as an animal-level risk factor (data not shown). Because of the low numbers of cows culled from small herds, it was considered inefficient to make additional herd visits to collect cull cow samples from small herds; therefore, only large herds were revisited. Five large herds had at least one positive cull cow, but they had no positive milk cows. Rather than limit the case definition to herds with positive milk cows only, we decided to use all available information, including results from cull cows, to more accurately classify these five herds. Although the variable herd size could not be evaluated as a risk factor because of differences in sampling protocol among herd size groups, herd size was included as a covariate in the model to account for the different sampling protocols.

A higher prevalence of *E. coli* O157 shedding was found in herds tested during the summer months compared with herds tested during the spring. A study in Washington also found the highest prevalence in summer *(11)*. Peak incidence of *E. coli* O157 infection in human beings is also in the summer *(8)*. Although cooking and eating habits during the summer (which include picnics and barbecues) may contribute to the seasonality of infections in humans, these factors cannot explain the increased shedding by cattle.

A "northern tier" effect has been described regarding outbreaks and sporadic cases of *E. coli* O157 in human beings *(8)*; however, a northern distribution was not observed in cattle in a feedlot study *(13)* or in this study. In fact, a higher prevalence was observed in herds located in the south. The higher prevalence observed in humans in the northern states may be due to more aggressive surveillance, culturing, and reporting in those states and not due to a higher level of animal shedding. It seems reasonable that regional patterns for VT-O157 shedding in cattle would not necessarily mirror that of human infection, since movement of cattle and meat distribution channels may span great distances.

Flushing alleyways with water to remove manure is a fairly common practice in large western dairy herds *(25)*.

Although effective in quickly removing manure from alleyways, this practice may distribute fecal flora throughout the cow housing environment, thus exposing large numbers of animals. Other researchers have cultured *E. coli* O157 from water and biofilm *(5, 10)*. There is evidence that *E. coli* O157 survives for long periods of time in water *(21, 23)*. Fourteen of the 19 farms in this study that flushed alleyways used recycled flush water. These farms may be providing contamination from the water in addition to spreading the VT-O157 already present in the manure in the alleyways. Whereas operations that flush alleyways account for only 21% (19 of 91) of all sample operations, they account for 59% (13 of 22) of the VT-O157–positive herds. Using the formula for attributable risk described by Bruzzi et al. *(1)*, over half (51.7%) of the cases could be eliminated by removing the effect of alley flush systems. The attributable risk assumes a cause-and-effect relationship and therefore should be interpreted cautiously when applied to a cross-sectional study design.

Analyzing the data using univariate analysis established that providing cows with individual waterers was protective, whereas operations using water tanks had an increased risk of VT-O157 shedding. The effect of water tanks could not be modeled with regard to region and season because water tanks were used by all herds in the southern region and all but two herds tested during the summer. A chi-square test (comparing water tanks with other watering systems) limited to northern herds tested during the spring was not significant. However, the sample size was reduced to only 54 herds. Thus, the power to detect an association was greatly reduced.

Operations that chlorinated cows' drinking water also had a lower shedding prevalence than other herds, but this difference was not statistically significant. Human health researchers recommend chlorination as a preventive measure against *E. coli* O157 exposure *(23)*. A protective effect of chlorination was not detected in this study as there may have been too few herds using this practice to detect a statistical association, or perhaps the amount of organic material contained in cows' drinking water is greater than what can be effectively neutralized by chlorination.

Feeding practices were hypothesized to affect the intestinal flora, including VT-O157 shedding. Previous studies have shown feeding corn silage to be associated with an increased risk of shedding VT-O157 *(14)*, whereas feeding clover and cottonseed were associated with a decreased risk of shedding *(7)*. However, none of the feed ingredients evaluated in this study were significantly associated with VT-O157 shedding. The univariate prevalences of VT-O157 by various feeding practices were not a good indication of risk because of the confounding effects of regional and seasonal feeding practices. For example, cottonseed was fed primarily to southern herds and in herds tested during the summer; they thus appeared univariately to be a risk factor for VT-O157 shedding. Similarly, meat–blood–bone meal was fed more frequently in northern herds and herds tested during the spring, and thus appeared univariately to be protective. These examples demonstrate the importance of adjusting for confounding factors when evaluating risk factors.

The small sample size limited our ability to detect risk

factors that were not of a very large magnitude. For example, spreading manure on land via irrigation, a practice employed by approximately one-quarter of the sampled herds, would require an odds ratio of at least 5 in order to be identified as a risk factor with 95% confidence *(6)*. A larger sample size would have increased the power to identify smaller but still important risk factors for VT-O157 shedding. However, we felt it was more important to use the available resources to obtain adequate numbers of samples within herd (thus improving the herd detection level) than to have more herds with fewer samples per herd (thus providing less confidence of herd detection).

Evaluating factors associated with VT-O157 is difficult because of low within-herd prevalence and sporadic shedding. Despite these obstacles, this study was helpful in understanding potential herd-level risk factors. Although a convenience sample was studied, the sample population resembled the national population in terms of the percentage of herds with Holstein cows. Study herds were more likely to participate in Dairy Herd Improvement Association programs and were more likely to be fed total mixed rations than were herds nationally. The average milk production was also higher than herds nationally *(24)*. These differences are most likely attributable to the larger size of participating herds compared with national averages. The diversity of management systems and regional and climatic influences of the study herds provided a unique opportunity to explore differences in VT-O157 shedding. Promising areas for research identified by this study relate to water management, such as use of alley flush systems and water tanks, as well as to the seasonal influence on VT-O157 shedding.

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